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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Dysregulated Wnt signaling is the cause of many human tumors, and induces breast cancer in mice. We have previously found that the initiating response to Wnt signaling is the accumulation of breast somatic stem cells. We propose that tumor development is related to this increase, and sought with this proposal to test whether the stem cells that arise in transgenic mice expressing Wnt effectors are more mutable than usual. This would explain why the preneoplastic glands invariably acquire mutagenic changes and progress to tumor development. In order to test this hypothesis, we cultured mammary clonagens (mammospheres) and proposed to test their mutability with chemical carcinogens. We found that, contrary to our prediction, mammospheres are almost completely absent in cultures of mammary epithelial cells that express Wnt effectors. Similarly, adherent clonagen numbers were reduced by 90%. We anticipated that their number would increase by 10-fold to reflect other results. We are investigating this phenomenon to determine whether this is due to inhibition of clonagen differentiation and expansion by ectopic Wnt signaling. This is likely to enable us to describe better the selective effects of Wnt signaling on mammary stem cells.

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Wnt-induced Progenitors: Are they Highly Mutable?

Introduction

Ectopic Wnt signaling is highly tumorigenic for mammalian epithelia. We have previously found that Wnt effectors induce mouse mammary stem cells to accumulate, and that this is closely correlated with the risk of subsequent tumor development (Liu et al., 2004). These somatic stem cells are a likely source of tumor precursor cells; they have the longevity that is required to fix and accumulate the genetic changes that confer a growth advantage on preneoplastic cells. In an effort to describe why increased stem cell number leads to tumor development, we proposed to test whether Wnt-induced mammary stem cells were more mutable than normal stem cells. In order to do this, we aimed to transfer normal and Wnt-induced mammary epithelial cell populations to suspension culture, to treat them with mutagenic carcinogens and to evaluate the fraction of mutant mammospheres (clonagens) that developed. Our hypothesis was that Wnt effectors change the predominant type of stem cell division from asymmetric self-renewal (associated with little genomic risk) to one that creates a population of long-lived but highly mutable stem cells that are likely to acquire oncogenic mutations.

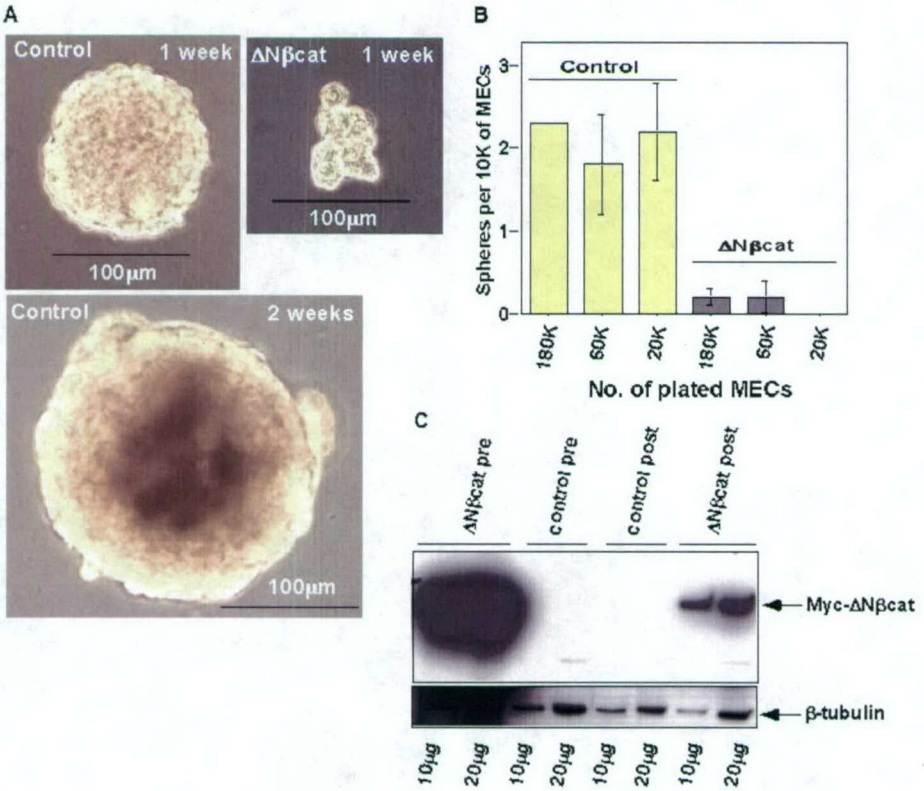
Body (Research Accomplishments)

Mammospheres that develop from mammary epithelial cell (MEC) populations at clonagenic frequencies may be analogous to the development of neurospheres in suspension culture of neural tissue. Neurospheres have stem cell activity in vivo and can be serially propagated for long periods. After dissociation of primary MECs from normal breast tissue, single cells form round clusters (consisting of ~150-300 cells) after 7-10 days of culturing, with a frequency of one sphere per 2,000 cells (Dontu et al., 2003).

We found that the number of mammospheres that develop in Wnt-induced mammary epithelial populations was reduced by 90%. This was surprising to us because we have already established that the stem cell activity of these populations is increased *in vivo* (using fat pad transfer) and that a subpopulation enriched in stem cells (SP fraction) is also increased (in ΔNβcat MECs by a factor of 15). This result did preclude us from measuring mutation rates as proposed, but has led us instead to evaluate why this happened, and to test key properties of mammosphere clonagens.

We cultured mammospheres from mammary epithelial cells (MECs) prepared from mammary glands from our two Wnt effector strains, [MMTV-Wnt1] and [MMTV-ΔNβcatenin] (ΔNβcat) transgenic mice (and from non-transgenic controls). The frequency of establishment of mammary clonagens from normal glands was similar to that published by Dontu et al. (2003). We observed that the number of mammospheres that formed from transgenic glands from Wnt effector strains (the example shown is from MMTV-ΔNβcatenin glands) was decreased by 90% (Fig. 1).

Fig 1. ΔNβcat-expressing MECs form mammospheres less efficiently than do control MECs. *MECs were isolated from 2-3-month-old FVB MMTV-*ΔNβcat *or control littermate mice, and cells were cultured at different densities (20,000-180,000 cells per 60 mm plate) on non-adhesive plates in serum-free culture). (A) Representative floating spheres grown from control or ΔNβcat-expressing cells following 1 or 2 weeks of culturing are shown. Not many well-rounded spheres were formed from ΔNβcat-expressing MECs, but some clusters did form as shown. (B) Quantitations of number of spheres per 10,000 input cells at various initial cell densities are shown. Spheres that are greater than 135μm in diameter were scored after 10 days of culturing; averages and standard deviations were derived from triplicate cultures. (C) Immunoblot analysis is shown for cell lysates from control and ΔNβcat-expressing cells prior to or subsequent of mammosphere culturing. ΔNβcat was detected by anti-Myc antibody, and anti-β-tubulin antibody was included as loading controls.*



Mammosphere formation depends upon a subtle balance of growth and differentiation to form semi-mature aggregates of >150 cells. We assumed therefore that if this balance was disturbed by the expression of Wnt effectors, that the suspended clonagens could exist but not be able to expand into mammospheres. To test whether this effect was an artifact of the suspension culture conditions, we changed the protocol to culture instead adherent clonagens that have also been related to stem cell activity (Alvi et al., 2002). This culture assay depends upon culture at low density on feeder layers and entirely different culture media, but the result is identical, that the clonagenic activity of Wnt-induced populations is reduced by 90%. We deduce therefore that this result is remarkably robust.

Prior to these studies, we anticipated that the clonagenic frequency would increase to reflect the observed increases in stem cell activity. Various other permutations of media and culture conditions (not described in detail here) did not change this result. This was problematic to pursuing our aim of examining the mutation rate of mammospheres in response to ENU.

In response, we have developed Rat-2 feeder cells expressing the soluble canonical Wnt signaling inhibitor, *dkk1*, and aim to test the clonagenic frequency of Wnt-1-expressing mecs. We reason that if Wnt-signaling is suppressing the ability of clonagens to grow out and differentiate (maintaining them instead as stem cells with little ability to survive in these media), then we will reveal the clonagenic activity on these feeder cells. These experiments are ongoing.

The premise of these studies is that the clonagens that expand into mammospheres are related to mammary stem cells. Mammospheres can be serially propagated, are enriched in SP progenitor fractions and give rise to both mammary lineages in culture (Dontu et al., 2003). However, given the paradoxical results we have obtained, we sought to validate the stem cell activity of mammospheres by transplantation back to fat pads. To date, we have not been successful. Another group has reported successful repopulation of fat pads using a mammosphere inoculum (Kurpioz and Hassell, personal communication), but their results suggest that the stem cell potency of these structures is low and incomplete. We continue to work on developing culture conditions that will give mammary stem biologists the same power as the Dexter cultures give to hematopoietic stem cell biologists. This is key to the functional evaluation of stem cell activity.

Key Research Accomplishments

- We have found that mammosphere formation is almost totally inhibited in MEC populations that express either a soluble or intracellular Wnt effector transgene.
- This result is also true for mammary clonagens isolated and cultured by distinct means, and we conclude that the result is highly robust.
- Although this precludes us from testing the mutability of these clonagens, it has led us to test *in vivo* whether mammosphere formation constitutes a valid assay of stem cell potential. We remain to be convinced that mammospheres retain sufficient stem cell potential to make them a suitable assay criterion.

Reportable Outcomes

Poster:
Keystone meeting; "Stem Cells and Development" January 2004.
B.L. Liu, S.S. Khwaja, and C.M. Alexander

PhD Thesis:
Bob L. Liu, University of Wisconsin-Madison, 2004.

Pre-Award Data:
Data will be used in applications to the NIH (*Alexander,* PI):
The role of stem cells in mouse mammary tumor development.

Conclusions

We continue to analyze the reasons for the paradoxical results we have obtained. Our experiments aim to describe the selective effects of Wnt effectors on growth or survival of progenitor cells, in order to find out why this response is linked to tumor development.

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Liu, Y., McDermott, S. P., Khwaja, S. S., and Alexander, C. M. (2004). The transforming ability of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc. Natl. Acad. Sci. USA *101*, 4158-4163.